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Comparison of early detection of *Fasciola hepatica* in experimentally infected Merino sheep by real-time PCR, coproantigen ELISA and sedimentation

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**Abstract**

Fasciolosis due to infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a significant global cause of livestock production loss. Infection is commonly diagnosed by a labour-intensive sedimentation and faecal egg count (FEC), which has limited throughput and is only applicable after completion of the 8–12 week pre-patent period (PPP). A commercially-available ELISA for the detection of coprological antigen (coproELISA) enables detection prior to the completion of the PPP and is suitable for diagnosis of larger sample sizes, although the sensitivity reported under experimental infection settings can be difficult to replicate in the field, particularly in cattle. A recently-published real-time PCR workflow for the sensitive detection of Fasciola spp. DNA in faecal samples provides increased sample throughput, although the point at which this technique is first able to diagnose infection remains unknown. Other tools for the molecular diagnosis of fasciolosis, such as conventional PCR and loop-mediated isothermal amplification (LAMP), have been shown to detect *F. hepatica* DNA as early as 1 week post infection (WPI). In this study, faecal samples were collected weekly from 10 experimentally-infected Merino lambs and subjected to diagnosis via traditional sedimentation, coproELISA and real-time PCR. Samples were first considered positive at 6–8 WPI by coproELISA, real-time PCR and sedimentation, respectively. At 9 WPI 100% of samples were positive by all three methods. To evaluate the capacity of the real-time PCR approach to detect infection prior to completion of the PPP, two methods of sample preparation were compared at 2 WPI: (i) 150 mg raw faecal samples and (ii) 3 g faecal starting volume prior to sedimentation and pelleting. Neither method of sample preparation yielded positive results at 2 WPI suggesting that DNA amplification by real-time PCR is associated with faecal egg load.

1. Introduction

Fasciolosis caused by infection with *Fasciola hepatica*, *Fasciola gigantica* or their hybrids is a zoontic parasitic disease of global importance (Torgerson and Macpherson, 2011). Up to 91 million people are considered at risk of infection and production losses are estimated to exceed US$2 billion/year (Keiser and Utzinger, 2005; McManus and Dalton, 2006). The parasite has an indirect life cycle, and the pre-patent period (PPP) is generally considered to take 8–12 weeks (Andrews, 1999; Brunsdon, 1967). During this time animal production may be affected due to the migration of immature stages through the liver, causing hepatitis, without externally detectable life cycle stages (Andrews, 1999). Associated production losses include decreased weight gain, anaemia, liver condemnation, reduced reproductive performance and, importantly in the case of acute fasciolosis, the potential for significant increases in animal mortality, often with minimal warning (Andrews, 1999).

Traditionally, ante mortem diagnosis has been performed by faecal egg counts (FEC) utilising either sedimentation or flotation techniques (Happich and Boray, 1969). These techniques have limited sensitivity for animals infected with liver flukes and are only of use after the PPP has passed (Happich and Boray, 1969). Recently, a commercial ELISA for the detection of fluke antigen in faeces (coproELISA) has enabled detection prior to the completion of the PPP (Mezo et al., 2004). However, issues have been identified regarding the decreased sensitivity of this approach in lighter infections (< 10 eggs per gram, EPG), particularly in cattle (George et al., 2017b; Gordon et al., 2012; Kajugu et al., 2015; Martinez-Sernandez et al., 2016). A serological ELISA is commercially available and in naïve animals provides the earliest indication of infection (Mezo et al., 2007). However, a prolonged
antibody response prevents this approach from detecting reinfection, limiting its application to surveillance of exposure (Mezo et al., 2007). A molecular approach utilising loop-mediated isothermal amplification (LAMP) has suggested that DNA may be detected in faeces from as early as one week post infection (WPI) in experimental studies (Martinez-Valladares and Rojo-Vazquez, 2016). Similarly, a real-time PCR-based workflow was recently published demonstrating high sensitivity in cattle faecal samples with low FECs (< 10 EPG) (Calvani et al., 2017). This approach has never been tested in an experimental infection setting and thus the point at which this technique is first able to detect Fasciola spp. DNA in faecal samples remains unknown (Calvani et al., 2017).

The present study aims to evaluate the earliest point of detection of F. hepatica in faecal samples in experimentally infected sheep using a recently described real-time PCR-based molecular workflow (for the detection of DNA from eggs), compared to a traditional sedimentation (for the detection of eggs) and the commercially-available coproELISA (for the detection of coproantigen released by metabolically active flukes). To determine the presence of F. hepatica DNA in faecal samples prior to egg shedding we tested raw and sedimented faecal samples at two weeks post infection.

2. Materials and methods

2.1. Experimental infection

All experimental infection was conducted at Yarrandoo R&D Centre (Queensland, Australia). Animals received a 10 month old Merino lambs were confirmed to be negative for F. hepatica prior to infection by sedimentation 12 weeks post removal from pasture. Animals were housed in group conditions in an indoor facility and fed a lucerne hay/oaten hay/straw/oats chalk mix, supplemented with a lucerne-based concentrate pellet. Water was provided ad libitum via the town supply system. Sheep were inspected at least daily for the duration of the sampling period. Animal ethics approval was provided by the Ethical Review Committee (approval number A3743). Each animal was infected per os with 250 Fasciola hepatica metacercariae generated at the Yarrandoo R&D Centre (New South Wales, Australia) for the purpose of strain maintenance. Weekly faecal samples were collected per rectum from 2 to 11 WPI. Four distinct Australian isolates of F. hepatica were used in order to maintain experimental strains; ‘Oberon’ (n = 2), ‘Numbugga’ (n = 2), ‘Palmers Oaky’ (n = 5) and ‘Bombala’ (n = 1). The ‘Oberon’ strain was isolated by the NSW Department of Primary Industries in 1999 and the remaining strains were isolated at the Yarrandoo R&D Centre (Fairweather, 2011; George et al., 2017). The ‘Numbugga’ strain was obtained from naturally infected goats near Bega NSW and the ‘Palmers Oaky’ strain was obtained from sheep grazing near Oberon NSW, both in 2014. The ‘Bombala’ strain was obtained from sheep grazing the Monaro region of NSW in 2016.

2.2. Sedimentation and faecal egg count

Eggs per gram of faeces (EPG) were determined by a standard sedimentation method with minor modifications as described in Calvani et al. (2017) (Happich and Boray, 1969). Briefly, after initial filtering through 270 μm nylon mesh, the modification consisted of three rounds of sedimentation, each three minutes in length, in successively smaller volumes of distilled water (250, 100 and 15 ml). The additional rounds of sedimentation served to remove vegetable matter and did not alter the percentage of eggs retained from the original method (Calvani et al., 2017; Happich and Boray, 1969).

Faecal samples were sedimented and counted in duplicate from 6 to 9 WPI and then once from 10 to 11 WPI. For each replication, 3 g of faeces was used. Duplicate sample EPGs are reported as the mean from two standard sedimentations. Duplicate counts during 6–9 WPI were employed to increase the sample volume to 6 g (2 × 3 g) in order to increase the sensitivity for samples with low counts (< 10 EPG). Samples were counted in duplicate backwards from 9 WPI until a minimum of two time points were negative for each animal. All morphological counts based on microscopic examination are henceforth referred to as mEPG.

2.3. DNA isolation and Fasciola hepatica real-time PCR

Faecal samples from 2 and 6–11 WPI were prepared and DNA isolated according to a previously published molecular diagnostic workflow available online at https://dx.doi.org/10.17504/protocols.io.jggcjtw (Calvani et al., 2017). Briefly, after initial sedimentation and egg-counting, the DNA in the resultant sediment was isolated using isolate Fecal DNA kit (Bioline, Australia) following the manufacturer’s recommendations. Samples were added to the 2 ml DNA isolation kit homogenisation tube with 750 μl of lysis buffer and homogenised in a high speed benchtop homogeniser at 6.0 m/s for 40 s (FastPrep-24, MP Biomedicals, Australia). For the 2 WPI samples, DNA was additionally isolated from 150 mg of raw faeces, homogenised as above and following the manufacturer’s recommendations (Bioline, Australia). DNA was eluted into 100 ul elution buffer (10 mM TrisCl buffer, pH = 8.5) and stored at −20 °C prior to amplification. To monitor DNA isolation efficiency and absence of PCR inhibition, 5 ul of DNA Extraction Control 670 (Bioline, Australia) was included and samples were assayed according to manufacturer’s instructions.

A TaqMan real-time PCR assay targeting F. hepatica ITS2 rDNA was utilised (oligonucleotides SSCPFaF [S0754]/SSCPFaR [S0755] and probe ProFh [S0770] FAM-BHQ1) and run in duplicate (Alasaad et al., 2011). A 10-fold dilution of the positive control of F. hepatica DNA served for quantification of egg estimate in EPG as previously described (Calvani et al., 2017). The EPG based on real-time PCR is referred to as qEPG.

All real-time PCR reactions were run on CFX96 Touch Real-Time PCR Detection System with the corresponding CFX Manager 3.1 software (BioRad, Australia) using SsoAdvanced Universal Probes Supermix (BioRad, Australia) according to the manufacturer’s instructions and cycling condition described previously (Calvani et al., 2017). Results were considered to be positive if both replicates displayed Cy values < 36. Each batch of DNA isolation was isolated with a blank sample (ddH2O) to detect contamination that may have occurred during the extraction process. Extraction Control samples with Cy values < 31 were considered not inhibited.

2.4. coproELISA

Faecal samples from 4 to 11 WPI were tested using a commercially available ELISA for the detection of Fasciola spp. coprological antigen in faeces (coproELISA) (BIK 201, Bio-X Diagnostics S.A., Belgium, batch number FASA17A20). Briefly, samples were thoroughly mixed with the kit dilution buffer (0.5 g + 2 ml) in 15 ml centrifuge tubes and allowed to sit overnight at 4°C to increase optical density (OD) readings of positive samples as recommended by Brockwell et al. (2013). Optical densities were read at 450 nm using a SpectraMax 250 plate reader (Molecular Devices, LLC., Sunnyvale, CA, USA). The OD of each corresponding negative well was subtracted from the individual sample ODs (Net OD). The Scaled OD was calculated by dividing the Net OD of the sample by the Net OD of the positive coproELISA controls. Samples were considered positive for F. hepatica antigen if the scaled OD was > 0.08.

3. Statistical analysis and data accessibility

Data was analysed in Microsoft Excel (2013) and visualized using GraphPad Prism version 6 (GraphPad Software, USA). Positive and
negative test results from each week were converted to binary values (1 = test positive, 0 = test negative) and significance was calculated via ordinary one-way ANOVA in GraphPad Prism version 6 (GraphPad Software, USA). The sedimentation technique was considered the gold standard in lieu of post-mortem examination due to the previously-reported high sensitivity of dual sedimentations for samples with low faecal egg loads (< 10 EPG) (Calvani et al., 2017). Raw data is available under the following DOI: https://http://dx.doi.org/10.17632/hsrngjtckj.1.

4. Results

All animals were successfully infected with their respective *F. hepatica* strains as determined by positive sedimentation, real-time PCR and coproELISA results at 9 WPI (Fig. 1). Result remained positive for all animals until 11 WPI, at which time sampling ceased. No differences in FEC, antigen detection or DNA amplification between strains was observed (data not shown).

Animals were first considered FEC positive at 8 WPI (7/10, 70%), with a mean FEC of 6 EPG (Fig. 2). FECs increased through week 9–11 with mean counts of 71, 236 and 586 EPG, respectively. Sheep 1 and sheep 10 (2/10 sheep, 20%) had FECs < 10 EPG at 9 WPI and gave the lowest FEC for the duration of observation (Fig. 2).

Animals were first considered positive at 6 WPI according to the coproELISA. Positive coproELISA results were seen in 30% of sheep at 6 WPI (95% CI = 0.10–0.61), 90% of sheep at 7 and 8 WPI (95% CI = 0.57 ≥ 0.99), and 100% of sheep at 9 WPI (95% CI = 0.68–1.00) (Fig. 2).

Animals were first considered positive at 7 WPI according to the real-time PCR. DNA was detected in 20% of faecal samples by real-time PCR at 7 WPI (95% CI = 0.05–0.52), 80% of sheep at 8 WPI (95% CI = 0.48–0.95) and 100% of sheep at 9 WPI (95% CI = 0.68–1.00) (Fig. 2).

Both the mEPG and qEPG were considered positive at 1EPG.

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Both the mEPG and qEPG were considered positive at 1EPG.
value = 0.0336 and < 0.0001, respectively), but not at week 8 (P-value = 0.8570).

To evaluate the presence of “free DNA” in the early stages of infection two methods of DNA isolation were applied to the 2 WPI faecal samples. DNA was isolated from 150 mg raw faeces or from the resultant sediment from a traditional sedimentation. Neither method of sample preparation yielded positive real-time PCR results at 2 WPI (data not shown).

5. Discussion

The capacity of molecular methods for the early diagnosis of fascioliasis have recently been explored as an alternative to the coproELISA, with claims that F. hepatica “free worm” DNA can be detected in faecal samples as early as 1–2 WPI (Martinez-Perez et al., 2012; Martinez-Valladares and Rojo-Vazquez, 2016; Robles-Perez et al., 2013). Several molecular tools, including conventional and nested PCRs, as well as loop-mediated isothermal amplification (LAMP), have been shown to detect F. hepatica DNA in faecal samples prior to completion of the PPP (<8 weeks) in experimental infections (Martinez-Perez et al., 2012; Martinez-Valladares and Rojo-Vazquez, 2016; Robles-Perez et al., 2013). It was proposed that successful amplification of DNA from faecal samples prior to the presence of F. hepatica eggs in faeces may be associated with cellular material from immature flukes, i.e. “free worm” DNA not contained within eggs (Martinez-Perez et al., 2012). Such “free worm” DNA potentially consists of cells sloughed from the integument of immature and adult flukes in response to the animal’s immune response (Martinez-Perez et al., 2012). A real-time PCR diagnostic workflow has previously demonstrated good correlation with morphological FEC results, but it was not known if these results were influenced by the presence of “free worm” DNA (Calvani et al., 2017). The current study aimed to evaluate the contribution of F. hepatica “free worm” DNA to the real-time PCR results in experimentally infected sheep faecal samples while also comparing the initial point of detection of infection with F. hepatica by real-time PCR to a traditional sedimentation and a commercially available coproELISA (Happich and Boray, 1969; Mezo et al., 2004).

Several studies comparing LAMP to real-time PCR for the diagnosis of a variety of parasitic and viral diseases have demonstrated agreement between the two techniques (Lin et al., 2012; Sugiyama et al., 2005; Wang et al., 2013). To evaluate the capacity of the real-time PCR to detect F. hepatica DNA without the presence of eggs in faeces we compared two methods of sample preparation on faecal samples collected 2 WPI: (i) isolation of DNA from 150 mg raw faeces and (ii) isolation of DNA from an initial volume of 3 g of faeces subjected to a traditional sedimentation and pelleting (Calvani et al., 2017; Happich and Boray, 1969). In contrast to a previous study that employed 0.5 g raw faeces for amplification of F. hepatica DNA with LAMP and conventional PCR, neither of our sample preparation methods yielded positive results at 2 WPI using real-time PCR (Martinez-Valladares and Rojo-Vazquez, 2016). The poor sensitivity of both conventional PCR and LAMP when diagnosing naturally infected animals, has been suggested as possibly due to insufficient starting volume of faecal material and efficiency of current DNA extraction methods (Ariffin et al., 2016). Our second approach addressed this issue by increasing the starting volume from the manufacturer-recommended 150 mg to 3 g prior to sedimentation and pelleting. Whilst this approach results in the concentration of eggs, increasing the sensitivity after completion of the PPP, it may inadvertently wash off any “free worm” DNA, resulting in false negative samples early on in the infection (Calvani et al., 2017). The earliest detection by real-time PCR using the sedimentation and pelleting approach for sample preparation occurred at 7 WPI and by 9 WPI all animals were considered PCR positive, suggesting that DNA detected by the current real-time PCR method is highly associated with the presence of F. hepatica eggs in faeces. A strong positive correlation between mEPG and qEPG (R² = 0.94) further suggests that our positive real-time PCR results are associated with faecal egg load, limiting its application to after completion of the PPP. Whether “free worm” Fasciola spp. DNA can be detected in faecal samples prior to the presence of eggs by other molecular approaches such as LAMP requires further scrutiny before they can be considered applicable in a diagnostic setting.

The traditional sedimentation results from the current study confirm the 8–12 week PPP for F. hepatica infection in Merino sheep and are in agreement with similar experimental infections of sheep, regardless of initial infective dose (Supplementary Table S1) (Brockwell et al., 2013; Flanagan et al., 2011; Martinez-Perez et al., 2012; Mezo et al., 2004; Valero et al., 2009). The majority of animals in this study (7/10) were positive by sedimentation as early as 8 WPI (mean FEC = 6 EPG) and all animals were positive by 9 WPI. Our results highlight the capacity of the sedimentation approach to identify infection in animals with low FECs (<10 EPG) (Supplementary Table S1) (Calvani et al., 2017; Happich and Boray, 1969). The high sensitivity in samples with low FECs was achieved through duplicate sedimentations (2 × 3 g faecal starting volume per sample), increasing the diagnostic sensitivity from 33% to 66% (Calvani et al., 2017; Happich and Boray, 1969). High sensitivity is important in both early and low-level infections, particularly in cattle where the large volume of faeces and low numbers of eggs make diagnosis by this method difficult. However, despite the observable increase in sensitivity, the increase in processing time required for a dual sedimentation/FECA approach hinders its applicability as a medium to large-scale diagnostic tool (Supplementary Table S2).

Detection of coprological antigen provides an approach for the diagnosis of Fasciola spp. infection capable of giving positive results prior to the completion of the PPP, with increased sample throughput compared to the traditional sedimentation (Supplementary Table S2) (Gordon et al., 2012; Mezo et al., 2004). During development of a commercially-available MM3 antigen-based coproELISA for the diagnosis of Fasciola spp. infection, initial reports stated detection of coproantigen as early as 5 WPI in sheep, with 100% of animals positive at 10 WPI (Mezo et al., 2004). Since commercialisation of this coproELISA, several experimental infection studies have reported similar results, with a slight delay in detection of animals infected with F. gigantica (Supplementary Table S1) (Flanagan et al., 2011; Martinez-Perez et al., 2012; Valero et al., 2009). Our results are in agreement with other experimental infections of sheep, with the first detection occurring at 6 WPI in 30% of experimentally infected sheep, and 100% of animals positive by 9 WPI (Supplementary Table S1) (Brockwell et al., 2013; Valero et al., 2009). Issues with the coproELISA have arisen in the diagnosis of lighter infections, particularly in cattle, where the dilution of coproantigen in faeces reduces the OD below the manufacturer’s recommended positive threshold (Brockwell et al., 2013; Calvani et al., 2017; Gordon et al., 2012; Kajugu et al., 2015; Novobolsky et al., 2012). It has been suggested that the large infective dose (≥200 metacercariae) used in many experimental infection studies may contribute to the disparity between the performance of the
coproELISA in experimental and natural infections (Gordon et al., 2012). An assessment of the capacity of the coproELISA against multiple life stages concluded that whilst it may be an appropriate tool for the monitoring of adult infections, it is not as reliable against immature stages of the parasite (George et al., 2017b). Subsequently, the performance of the coproELISA in the current study may not necessarily reflect its diagnostic sensitivity in a natural infection setting.

6. Conclusion

Both the real-time PCR and coproELISA are more convenient and allow greater sample throughput than the traditional sedimentation approach, particularly if dual sedimentation/FECs are required for increased sensitivity (Supplementary Table S2) (Calvani et al., 2017; George et al., 2017b; Gordon et al., 2012). An additional benefit of the DNA isolation includes the prolonged storage of samples for multiple uses, such as the potential for incorporation into existing diagnostic PCR panels such as those targeting sheep nematodes (Roebber et al., 2012; Roebber et al., 2017). A lack of detection of “free worm” DNA limits the capacity of the real-time PCR approach to diagnosis after completion of the PPP. Despite the fact that the real-time PCR failed to detect positive samples at 2 WPI, there was no significant difference between the diagnostic capacity of the three methods (sedimentation, coproELISA and real-time PCR) at 8 WPI in an experimental infection setting.

Conflict of interest statement

SG is a paid employee of ELANCO Animal Health, Australia. All other authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetpar.2018.01.004.

References